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Purification, crystallization and preliminary X-ray characterization of a haemagglutinin from the seeds of *Jatropha curcas*

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The plant *Jatropha curcas* (Euphorbiaceae) is an important source of biofuel from the inedible oil present in its toxic seeds. The toxicity arises from the presence of curcin, a ribosome-inactivating protein showing haemagglutination activity. In this communication, the purification, crystallization and preliminary X-ray characterization are reported of a small protein isolated from *J. curcas* seeds with a molecular mass of ∼10 kDa that agglutinates rabbit erythrocytes. The protein was crystallized using the hanging-drop vapour-diffusion method and also by the microbatch method in 72-well HLA plates, using PEG 8000 as the precipitant in both conditions. X-ray diffraction data collected from the rod-shaped crystals were processed in the orthorhombic space group $P2_12_12_1$. The crystals diffracted to 2.8 Å resolution at 103 K.

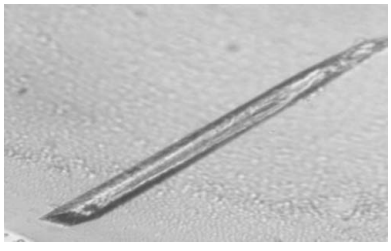
1. Introduction

Jatropha curcas L. is an interesting member of the Euphorbiaceae family of plants and has become commercially important owing to the use of its seed oil as a biofuel (Abdulla *et al.*, 2011). *J. curcas* seeds are toxic because of the presence of curcin, a ribosome-inactivating protein (RIP) similar to the toxic proteins ricin and abrin from *Ricinus communis* and *Abrus precatorius*, respectively (Stirpe *et al.*, 1976). In this report, we describe the purification, crystallization and preliminary X-ray characterization of a small protein isolated from the seeds of *J. curcas* with a mass of about 10 kDa that shows haemagglutination activity when tested on rabbit erythrocytes.

2. Materials and methods

2.1. Isolation and purification

J. curcas seeds were collected from Gujarat Forest Development Corporation, Gandhinagar, Gujarat, India. 100 g of seeds were decorticated to remove the outer cover and the powdered kernels were defatted with petroleum ether and stirred overnight together with 500 ml 1× PBS pH 7.4 at 277 K; the suspension thus obtained was clarified by filtration through double-layer muslin cloth and centrifugation at 6000 rev min^{-1} for 30 min at 277 K to obtain a clear solution. 60% ammonium sulfate was slowly added to this solution with stirring to precipitate the total protein content, which was further dissolved in a minimal volume of pre-cooled 1× PBS pH 7.4 and dialyzed against the same buffer 4–5 times using a dialysis bag to remove the salt. The crude protein solution obtained as described above was loaded onto a Sephacryl S-300 column (2 × 120 cm), eluted with 1× PBS buffer pH 7.4 using an ÄKTAprime purification system (GE Life Sciences) and fractionated. The fractions that showed agglutination activity with rabbit erythrocytes were pooled (elution volume between 150 and 180 ml), dialysed against 10 m*M* Tris–HCl pH 9 and loaded onto a QFF anion-exchange column (GE Life Sciences) pre-equilibrated with the same buffer. A gradient elution with 10 m*M* Tris–HCl pH 9 containing 1 *M* NaCl was carried out to elute the protein and its purity was checked using SDS–PAGE (Laemmli, 1970).



2.2. Agglutination assay

The assay for haemagglutination activity was performed using a microtitre plate in which 50 µl 1% rabbit erythrocyte suspension in 1× PBS and 50 µl serially diluted protein solution in 1× PBS with an initial concentration of 0.8 mg ml^{-1} were added to different wells and the mixtures were incubated at room temperature for 1 h. A negative control was also used in which only buffer without protein was added to the rabbit erythrocyte suspension. The end point in each case was non-formation of a button (sedimented cells) in the case of agglutination and *vice versa*, which was visually observed. Haemagglutination was expressed in haemagglutination units (HU; defined as the reciprocal of the highest dilution showing detectable haemagglutination) and the specific activity was calculated by dividing the activity in HU by the protein concentration (in mg ml^{-1}) showing this activity. Rabbit erythrocytes were used for haemagglutination tests throughout unless stated otherwise. N-terminal sequencing of the protein was performed at RIKEN, Japan.

2.3. Crystallization

Crystallization conditions were screened by the hanging-drop vapour-diffusion method using the Basic Crystallography Kit for proteins (Sigma). Hanging drops were set up on siliconized cover slips by mixing 3 µl reservoir solution with 1 µl protein solution (4 mg ml^{-1} protein in 1× PBS pH 7.5) and equilibrated by vapour diffusion against 500 µl reservoir solution. Crystallization was also set up using the microbatch method in 72-well HLA plates (Nunc) by mixing 1 µl protein solution with 3 µl precipitant layered with a 1:1 mixture of silicone oil and paraffin oil.

2.4. Data collection and processing

A single crystal chosen from the microbatch setup was mounted using a cryoloop and cooled in a liquid-nitrogen stream at 103 K produced by an X-stream (Rigaku). No separate cryoprotectant was used. The X-ray diffraction data were collected on an R-AXIS IV^{++} image plate using Cu $K\alpha$ radiation generated by a Rigaku rotating-anode X-ray generator operated at 50 kV and 100 mA and equipped with a confocal mirror focusing system. The diffraction data, which consisted of 360 images spanning 180° with 0.5° oscillations, were processed and scaled using *CrystalClear* software v.2.0 (Rigaku–MSC).

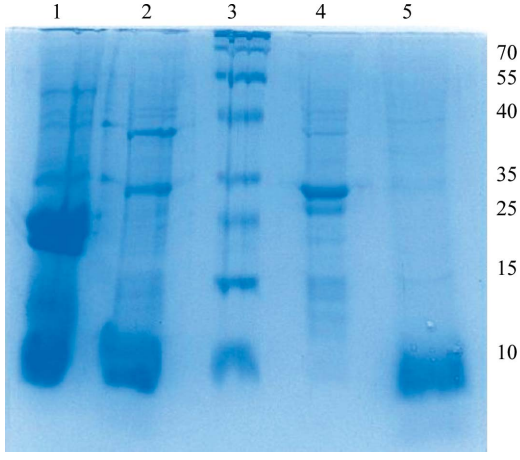


Figure 1
SDS–PAGE (15%). Lane 1, total protein content isolated using ammonium sulfate precipitation from a clarified 1× PBS suspension of *J. curcas* seeds; lane 2, the pooled fractions from Sepharose S300 showing haemagglutination activity; lane 3, protein molecular-weight marker (labelled in kDa); lane 4, the flowthrough from the QFF column; lane 5, purified haemagglutinin.

3. Results and discussion

When purified using a Sephacryl S300 column, the total crude protein solution isolated from 100 g *J. curcas* seeds gave partially purified peaks at elution volumes between 150 and 180 ml that showed haemagglutination activity with rabbit erythrocytes. When loaded onto a QFF anion-exchange column and eluted with increasing concentrations of NaCl, these pooled fractions separated into different components. The fraction eluted using an NaCl concentration of 44–56% showed a single band at about 10 kDa for the purified protein (Fig. 1) on SDS–PAGE and agglutinated rabbit erythrocytes with a specific activity of 25 HU mg^{-1}. The mass was further confirmed using mass spectrometry, and N-terminal sequencing of the protein showed the following sequence for the first ten residues: VRDIRKKEAE.

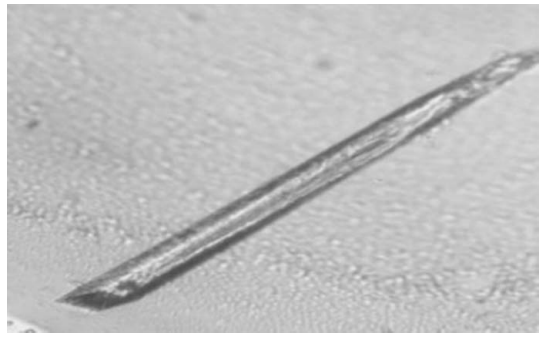


Figure 2
Needle-shaped orthorhombic crystal of *J. curcas* haemagglutinin grown using 0.1 *M* Tris–HCl pH 8.5 buffer, 8%(*w*/*v*) PEG 8000 as precipitant.

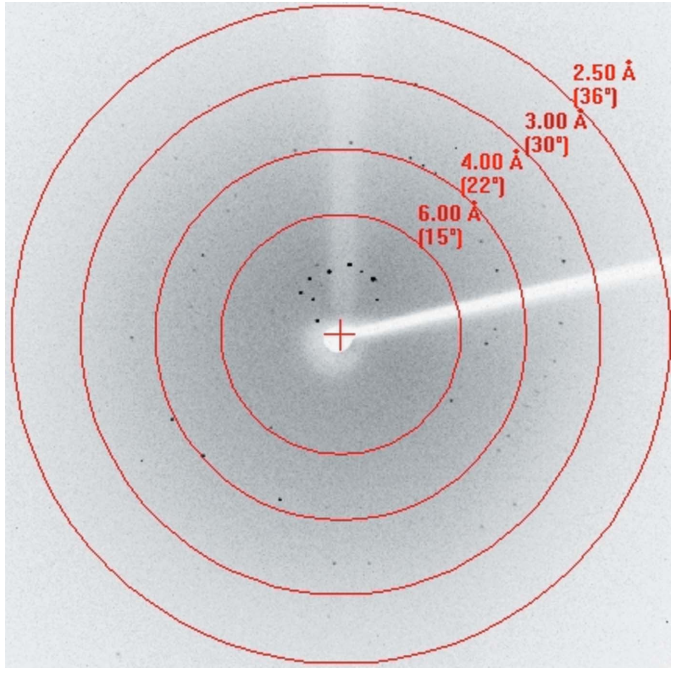


Figure 3
Diffraction image of a haemagglutinin crystal showing various resolution shells. The oscillation was 0.5° and the crystal-to-detector distance was 200 mm.

Table 1
Diffraction data statistics for *J. curcas* haemagglutinin.

Temperature (K)	103
X-ray source	Cu $K\alpha$
Wavelength (Å)	1.542
Resolution range (Å)	33.27–2.80 (2.90–2.80)
Total reflections	11864
Unique reflections	1928
Completeness (%)	95.6 (100)
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 32.54, b = 43.89, c = 51.02
Average multiplicity	6.15 (6.76)
$R_{\rm merge}$†	0.075 (0.213)
$\langle I/\sigma(I)\rangle$	25.1 (10.9)
Matthews coefficient (Å^3 Da^{-1})	1.92
No. of molecules in asymmetric unit	1
Solvent content (%)	35.9

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

Several crystal forms grew within a day of setting up the crystallization experiment and two particularly promising conditions that gave needle-shaped crystals were conditions No. 22 (0.2 *M* sodium acetate, 0.1 *M* Tris–HCl pH 8.5, 30% PEG 4000) and No. 36 (0.1 *M* Tris–HCl pH 8.5, 8% PEG 8000) of the Sigma kit. These conditions were further optimized. Finally, long needle-shaped crystals (Fig. 2) were obtained from 0.1 *M* Tris–HCl pH 8.5, 8%(*w*/*v*) PEG 8000 using the same setup as described in §2. Similar crystals obtained using the microbatch method under the same conditions were used for X-ray data collection at cryotemperature.

The orthorhombic crystals belonged to space group $P2_12_12_1$ and diffracted to 2.8 Å resolution at 103 K (one of the oscillation images is shown in Fig. 3); data-processing statistics are shown in Table 1. A total of 11 864 reflections were collected, of which 1928 were unique; the completeness of the diffraction data was 95.6%. The calculated Matthews coefficient (Matthews, 1968) of 1.92 Å^3 Da^{-1} corresponded to a solvent content of 35.9% for one molecule in the asymmetric unit.

Previous studies have reported the isolation of curcin with a molecular mass of 28.1 kDa and haemagglutination activity (Stirpe *et al.*, 1976; Lin *et al.*, 2002, 2003, 2010) and of another haemagglutinin with a molecular mass of 660 kDa composed of two different subunits of molecular masses 23.5 and 11.5 kDa (Cano Asseleih & Plumbley, 1989).

The haemagglutinin isolated by us differs from those isolated previously, as shown by SDS–PAGE. The *J. curcas* genome has recently been sequenced (Sato *et al.*, 2011) and a survey of the online database (http://www.kazusa.or.jp/jatropha/) shows the presence of many small proteins in the genome such as malectin, curculin-like protein *etc.* Further work on structure determination of the protein is in progress.

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